**Title**: Thioredoxin reductase (TrxR) as a novel target for antifilarial drug development

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## Detailed research proposal:

### Introduction

Lymphatic filariasis (LF) is a deforming and disabling disease caused by roundworm parasites, transmitted by mosquitoes (Krentel *et al.,* 2013). Like many other neglected tropical diseases (NTDs), LF disproportionately affects vulnerable populations and perpetuates existing relationships between disease and poverty (Hotez *et al.,* 2009). The Global Program to Eliminate Lymphatic Filariasis (GPELF) has made noteworthy progress in many countries. The key strategy of the elimination program comprises repeated annual rounds of mass drug administration (MDA) to populations at risk of acquiring the infection with diethylcarbamazine (DEC) plus albendazole outside of Africa and with ivermectin plus albendazole in Africa (Ottesen *et al.,* 1999). MDA reduces microfilariae (Mf) in human blood which is the major objective of the program in order to interrupt transmission. Repeated rounds of MDA are required because current regimens fail to kill all adult worms and completely clear Mf following single-dose treatments. The filarial parasites are long lived and are capable of surviving in host's environment by employing varieties of evasion strategies and defence mechanisms (Schmid-Hempel, 2009). They possess a strong anti-oxidant system to protect themselves from hostile environment of the host. These parasites possess different antioxidant enzymes like superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione-S-transferase (GST), glutathione reductase (GR) and thioredoxin reductase (TrxR).

One of these enzymes, TrxR plays an important role in the survival of the parasites (Henkle-Duhrsen and Kampkotter, 2001).TrxR is a homodimeric flavo oxidoreductase critically involved in regulating the cellular redox homeostasis, growth, and differentiation, having ubiquitous distribution such as in bacteria, plants, fungi, insect (*Drosophila melanogaster*), the nematode (*Caenorhabditis elegans*), malaria parasite (*Plasmodium falciparum*) and mammals supporting its conserved behavior. Its successful involvement in different diseases (Leitsch et al., 2016; Becker et al., 2003) makes TrxR a highly tempting drug target. This dimeric TrxR belongs to pyridine nucleotide disulfide oxidoreductase family (Mustacich and Powis, 2000), comprising of a FAD prosthetic group, a NADPH binding site and a redox active disulfide (Becker *et al.,* 2000). Existing reports indicate the appearance of two distinct subtypes, a high molecular weight (H-TrxR) (~55kDa) thiordoxin reductase and

a low molecular weight (L-TrxR) (~35 kDa) thioredoxin reductase (Williams Jr. *et al.*, 2000). On contrary to the cysteine contains TrxR present in various organisms, mammalian and cytoplasmic TrxR of nematode *C. elegans* possess selenocysteine in place of cysteine residue.

A wide range of TrxR substrates include proteins and molecules of low molecular weight (Becker *et al.*, 2000). Apart from the size, the charge and the polarity of the substrates act in this issue. The notion has been already conceived regarding the catalytic mechanism that larger substrates reacts in the C terminal active site of the TrxR and the smaller one utilizes the flavin or the internal catalytic cysteines (Becker *et al.*, 2000). Mammalian TrxR catalyzes a range of low molecular weight substrates having disulfides which includes 5, 5′-dithiobis-(2-nitrobenzoic acid) (DTNB), lipoic acid and lipoamide. Nondisulfide substrates viz. selenite, quinone compounds and ascorbic acid are also used. This broad range of substrate specificity largely depends on the accessible C terminal selenenylsulfide/ selenothiol active centre and the N terminal FAD/-Cys-Val-Asn-Val-Gly-Cys redox centre that directly reduce small substrates not depending upon the usual Sec containing active site (Rackham *et al.*, 2011). Because of the broad substrate specificity H-TrxR are concerned in many metabolic pathways. TrxR also utilizes selenodiglutathione, NK-lysin, Lipoic acid (lipoate disulfide), Ascorbyl free radicals, S-Nitrosoglutathione (GSNO) (Becker *et al.*, 2000).

The presence of TrxR in the microfilarial and adult stages of filarial parasite, *Setaria cervi* has been reported by Yadav *et al.*, 2013. But the specific role of TrxR in the filarial parasites is still not transparent. However, significant role of TrxR in the survival of the filarial parasites have been elucidated by Tiwari *et al.*, 2014.

Targeting through inhibitors of TrxR enzyme can be a novel approach to fight against varieties of diseases. A large number of synthetic chemicals are known inhibitors of TrxR from diverged sources. Auranofin, which is a known drug for rheumatoid arthritis has been successfully repurposed against lymphatic filariasis and onchocerciasis infection (Bulman *et al.*, 2015).

**Proposed objectives**

i. To identify the presence of TrxR in filarial nematodes *in situ* and optimization of the extraction parameters of TrxR with highest biochemical activity.

ii. To purify, characterize and sequence the filarial TrxR.

iii. To examine the effect of conventional and novel antifilarial compounds on TrxR for macrofilaricidal activity.

iv. To investigate the interaction of purified parasitic TrxR with host immune cells.

**Experimental design**

*Ethical clearance*

The protocols for this study will be approved by the Institutional Animal Ethics Committee, (CPCSEA) Govt. of India (1819/GO/Ere/S/15/CPCSEA), and the institutional Human Ethical Committee Visva-Bharati.

*Collection of parasites*

This study will be conducted using adult gravid females and males of bovine filarial nematode parasite *Setaria cervi*, a WHO recommended model for filarial research (Ahmad *et al.,* 2007; Mukherjee et al. 2014, Gucchait *et al.,* 2018). The studies regarding the microfilariae of *Wuchereria bancrofti* (the human filarial nematode) will be conducted through nocturnal blood sampling conducted in municipality or panchayat approved filariae endemic villages in and around Santiniketan. Full proof strategies are to be made for obtaining adults of *W. bancrofti*.

*Response Surface Methodology (RSM) guided optimization of extraction condition and localization of Thioredoxin reductase (TrxR)*

Response surface methodology (RSM) with Box-Behnken design has been a interesting tool for optimization of process parameters amongst a number of statistical tools due to its documented advantages in developing suitable design matrix with lesser number of experiments, cost effectiveness and accuracy (Mukherjee *et al.,* 2014; Mukherjee *et al.,* 2014; Liu *et al.,* 2017).

*Preparation of parasite lysates, gel electrophoresis and immunoblotting*

Adult parasites will be collected and dissected to isolate the microfilariae and oocytes. Final volume of the extraction buffer (100 mM PBS containing 1 M potassium dihydrogen phosphate and 1 M di-Potassium hydrogen phosphate, pH 7.5) will be adjusted according to 1 g weight. Further homogenization will be made using the hot plate stirrer for the requisite period of time and temperature. After stipulated time of extraction, homogenates will be collected in centrifuge tube and will be centrifuged at 10000g rpm, 4 ◦C for 20 min. Supernatant will be collected for the final assay.

*Assay of TrxR activity*

Reduction of 5, 5'-dithio-bis-[2-nitrobenzoic acid] (DTNB or Ellman’s reagent) to 2-nitro-5-thiobenzoate by the parasite lysate in the presence of NADPH will be measured spectrophotometrically at 412 nm. One unit of enzyme activity is defined as the production of 2 μmol of 2-nitro-5-thiobenzoate per μmole of NADPH oxidized per minute (ε 412 nm 13.6 $mM^{-1}$ $cm^{-1}$) (Yadav *et al.,* 2013).

*Localization of TrxR on parasite*

Immune localization of the enzyme in different developmental stages will be detected using immunofluorescence following the methods of Mukherjee *et al.,* 2017 and Haegeman *et al.,* 2009.

*Purification, characterization and sequencing of TrxR*

Purification of the enzyme of interest will be done following bioactivity guided purification process and pure protein will be sequenced through MALDI-TOF/MS analysis.

*Screening of conventional and novel antifilarial compounds on TrxR*

Purified and biochemically characterized TrxR will be used as drug target for the treatment of lymphatic filariasis. Several previously reported standard antifilarial drugs, synthetic compounds and their analogs will be used to evaluate the interaction between compounds and the purified enzyme.

*Studies on the immune interactions between the purified TrxR and host*

In order to investigate the immunological potential of the purified TrxR, it will be used to understand the interactions between the host immune cells in terms of pathology and protection. Pro inflammatory cytokines viz. TNFα, IL6, IL1β and anti inflammatory cytokines viz. IL10, TGFβ will be estimated for determining the effect of TrxR in altering the immune homeostasis of filarial infected host.

**Probable outcome**

The study will provide an insight towards a biochemical target which will be effective in treating lymphatic filariasis. Currently available remedies are effective only in microfilarial (mf) stage (Shenoy, 2008), macrofilaricidal agents are in need for successful elimination of lymphatic filariasis. Purified protein can be used as a vaccine for long term protection against this neglected tropical disease.

After completion of the study research articles will be published in peer review referred journals.

**Keywords**: Lymphatic filariasis; Antioxidant; Thioredoxin reductase (TrxR); Drug target, Drug development.

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